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Title: Determination of Ivermectin, Doramectin, and Moxidectin by HPLC		
Revision: 02	Replaces: CLG-AVR.01	Effective: 12/22/2006

Contents

A. INTRODUCTION

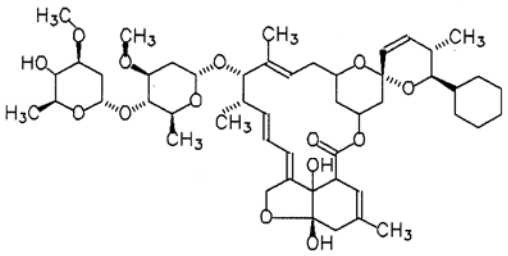
1. Theory

Moxidectin, ivermectin and doramectin are potent anthelmintics used in food animals to control parasitic infections. Moxidectin, ivermectin and doramectin are extracted from tissue with acetonitrile; extraneous substances are removed using alumina chromatographic cleanup. The analytes are determined by HPLC after formation of fluorescent derivatives with trifluoroacetic anhydride/1-methylimidazole.

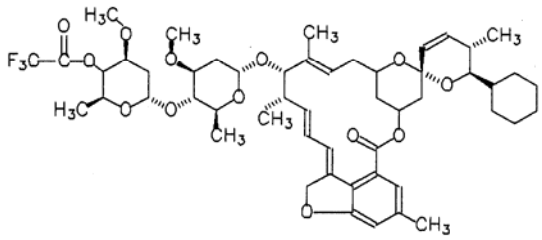
2. Applicability

The method is applicable to determination of Moxidectin, Ivermectin, and Doramectin in liver and muscle of bovine, ovine, porcine, caprine and equine species at ≥ 7.5 ppb.

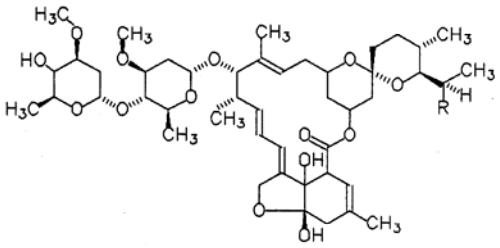
3. Structures



Doramectin

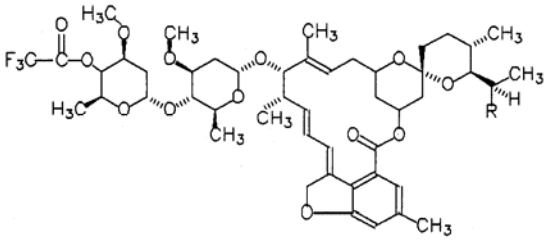


Doramectin Derivative



Ivermectin

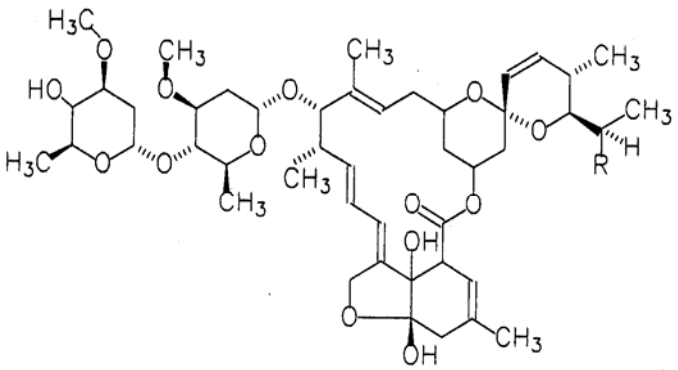
R = C_2H_5 for H_2B_{1A}
R = CH_3 for H_2B_{1B}



Ivermectin Derivative

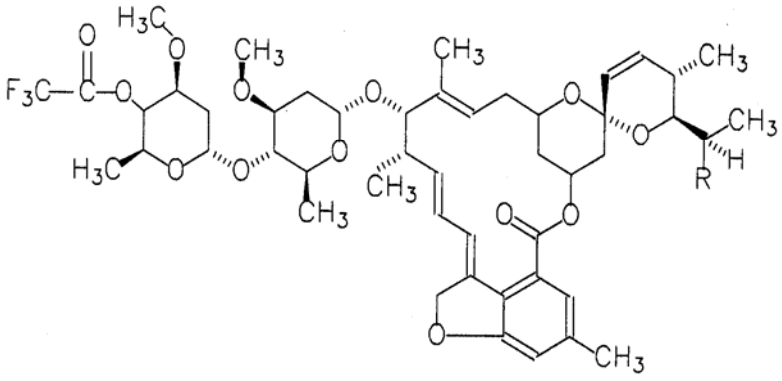
R = C_2H_5 for H_2B_{1A}
R = CH_3 for H_2B_{1B}

3. Structures (cont.)



Abamectin

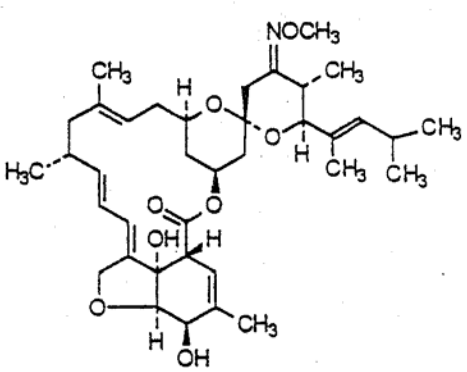
R = C_2H_5 for H_2B_{1A}
R = CH_3 for H_2B_{1B}



Abamectin Derivative

R = C_2H_5 for H_2B_{1A}
R = CH_3 for H_2B_{1B}

3. Structures (cont.)



Moxidectin

B. EQUIPMENT

Note: Equivalent apparatus and/or instrumentation may be substituted.

1. Apparatus

   a. N-EVAP - Model 112, Organomation Assoc. Inc.

   b. Centrifuge - Sorvall model T-6000B, DuPont Co.

   c. Mechanical shaker - Eberbach model 610 equipped with shaker box model 6040, Thomas Scientific.

   d. Vortex mixer - Fisher Scientific.

   e. Extraction columns - Fisher Scientific Prep Sep-R (empty), Cat. No. P449R, Fisher Scientific.

   f. 50 mL screw cap centrifuge tubes - Cat. No. 05-558-12B, Fisher Scientific.

   g. 50 mL polypropylene centrifuge tubes - Cat. No. 222-3937-G80, Evergreen Scientific International Inc.

   h. EDP Plus Micropipet - Rainin Instruments Inc.

   i. Eppendorf Pipettor - Cat. No. 4789, Brinkman Instrument Inc.

   j. Eppendorf Combitips - 5.0 mL and 12.5 mL, Brinkman Instrument Inc.

   k. SPE Cartridges - Place a small silanized glass wool plug into the neck of a 5.75" disposable transfer pipet. Add 0.1 ± 0.01 g C18 bulk packing material into the

disposable pipet. Tap gently to settle.

l. Glass test tubes - 16 x 100 mm, 20 x 150 mm, and 12 x 75 mm, Fisher Scientific.

2. Instrumentation

a. Liquid Chromatography System

i. Agilent 1100 Series Quaternary Pump, Agilent Technologies, Inc.

ii. Agilent 1100 Series Autosampler, Agilent Technologies, Inc.

iii. Agilent 1100 Series Vacuum Degasser, Agilent Technologies, Inc.

iv. Agilent 1100 Series Fluorescence detector, Agilent Technologies, Inc.

v. Agilent 1100 Series Column Compartment, Agilent Technologies, Inc.

vi. Zorbax ODS 4.6 mm x 15 cm C18 analytical column, Agilent Technologies, Inc.

vii. Brownlee Columns Spheri-5 RP-18, 30 mm x 4.6 mm guard column, 5 micron particle size, Perkin Elmer.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted if necessary.

1. Reagents

a. Acetonitrile LC Grade

b. Alumina-Neutral type WN-3, Activity grade 1, Sigma Chemical Co. Dry at 135 ± 5 °C for at least 24 hours prior to use.

Prepare deactivated alumina for column chromatography. Alumina should be 12% deactivated For example: Add 24 g distilled/deionized water to 176 g alumina. Mix by shaking until there are no visible lumps. Store deactivated alumina at room temperature in a tightly closed container. Use within one week after opening.

Prepare alumina columns by weighing 2.0 ± 0.2 grams of deactivated alumina into an empty Prep-Sep column.

c. 1-Methylimidazole - redistilled (99+ %), Cat. No. 33,609-2, Aldrich Chemical Co.

d. Trifluoroacetic anhydride (TFAA) - (99+ %), Cat. No. 10,623-2, Aldrich Chemical Co.

e. Methanol - LC Grade.

f. Water - HPLC grade.

2. Solutions

   a. 1-Methylimidazole - 1:1 v/v 1-methylimidazole/acetonitrile:

      Add 1 part acetonitrile to 1 part 1-methylimidazole.

      For forty samples, add 5 mL of acetonitrile to 5 ml of 1-methylimidazole and mix.

   b. TFAA - 1:1 v/v trifluoroacetic anhydride/acetonitrile:

      Add 1 part of acetonitrile to 1 part trifluoroacetic anhydride.

      For forty samples, add 5 mL of acetonitrile and 5 mL of trifluoroacetic anhydride and mix.

D. STANDARDS

1. Source

   a. Ivermectin standard, Cat. No. L-640,471-076P004, Merck Manufacturing Division, West Point, PA.

   b. Abamectin standard, Cat. No. L-676,863-038A003, Merck Manufacturing Division, West Point, PA.

   c. Doramectin standard, Cat. No. UK-67,994, Pfizer, Groton, CT.

   d. Moxidectin standard, Cat. No. 301423, Fort Dodge Animal Health.

2. Preparation

   a. Stock solution:

      Follow manufacturer's instructions accompanying standards to obtain a stock solution of approximately 125 ± 1 µg/mL in acetonitrile.

   b. Fortification solution:

      Make a 1:250 dilution to obtain a fortification solution of 0.5 µg/mL in acetonitrile.

      Add 1 mL each of doramectin, ivermectin and moxidectin stock solutions to a 250 mL flask and bring to volume with acetonitrile and mix.

3. Storage and Stability (if not included with preparation)

   a. Store stock solution in freezer < -10 ºC.

   b. Fortification solutions may be stored at room temperature.

   c. Stability:

      Fortification solution: 90 days.

      Stock solution: 1 year.

E. SAMPLE PREPARATION

Process samples of liver and muscle until homogeneous. All samples are stored refrigerated or frozen until analyzed.

Note: Remove excess fat from tissue before homogenization.

F. ANALYTICAL PROCEDURE

1. Sample extraction
   a. Weigh 2.5 ± 0.2 g ground tissue into a 50 mL polypropylene centrifuge tube.
   b. Add 8 mL acetonitrile and vortex for 30 sec.
   c. Fortify each sample with 150 µL of 0.5 µg/mL (equivalent to 30 ppb) abamectin internal standard solution. Fortify moxidectin, ivermectin and doramectin recoveries with appropriate volume of 0.5 µg/mL fortification solution depending on species analyzed.
   d. Centrifuge for 3 min at 1500 RPM.
   e. Pour acetonitrile eluent through deactivated alumina column and collect eluate in a 50 mL glass centrifuge tube.
   f. Repeat extraction with additional 8 mL acetonitrile, centrifuge and decant through alumina column combining eluents.
   g. Evaporate acetonitrile under a gentle stream of dry nitrogen or dry air at 65 °C.
   h. Reconstitute the dried sample from step (g) using 0.5 mL acetonitrile. Vortex to mix.
   i. For muscle tissue - Add 2 mL acetonitrile and proceed to step (m).

      For liver tissue - Prepare SPE C18 cartridge by placing a small silanized glass wool plug into the neck of a 5.75" disposable pipet. Add 0.1 g of C18 bulk packing material into the disposable pipet on top of the glass wool. Tap gently to settle C18. This SPE clean-up step eliminates co-extractants that interfere with quantitation.
   j. Pre-wet the SPE cartridge with 1.0 mL acetonitrile. Discard the wash.
   k. Load the 0.5 mL sample from step (h) onto the wet SPE cartridge. **(The columns must not dry out at any time or the Avermectin recoveries will be low).** Collect the eluent in a test tube.
   l. Add 2 mL of acetonitrile to the sample tube and mix. Add to the SPE column. Collect the eluent in the same container as the initial 0.5 mL eluent.

      Note: Properly dispose of the SPE column.
   m. Add 200 µL 1-methylimidazole/acetonitrile reagent to the eluent and vortex at

least 10 sec.

n. Add 200 µL TFAA/acetonitrile reagent and vortex at least 10 sec.

o. Allow the sample to derivatize in the dark for a minimum of 15 minutes before HPLC analysis.

Note: Derivatized samples decompose on exposure to strong light.

p. Inject on HPLC.

2. Instrumental Settings

Note: System may be adjusted to insure optimum response.

a. Mobile phase: 3:97 v/v water/methanol.

b. Flow rate: 1.8 mL /min.

c. Column temp.: 30 °C.

d. Injection vol.: 50 µL - As determined by detector/integrator conditions.

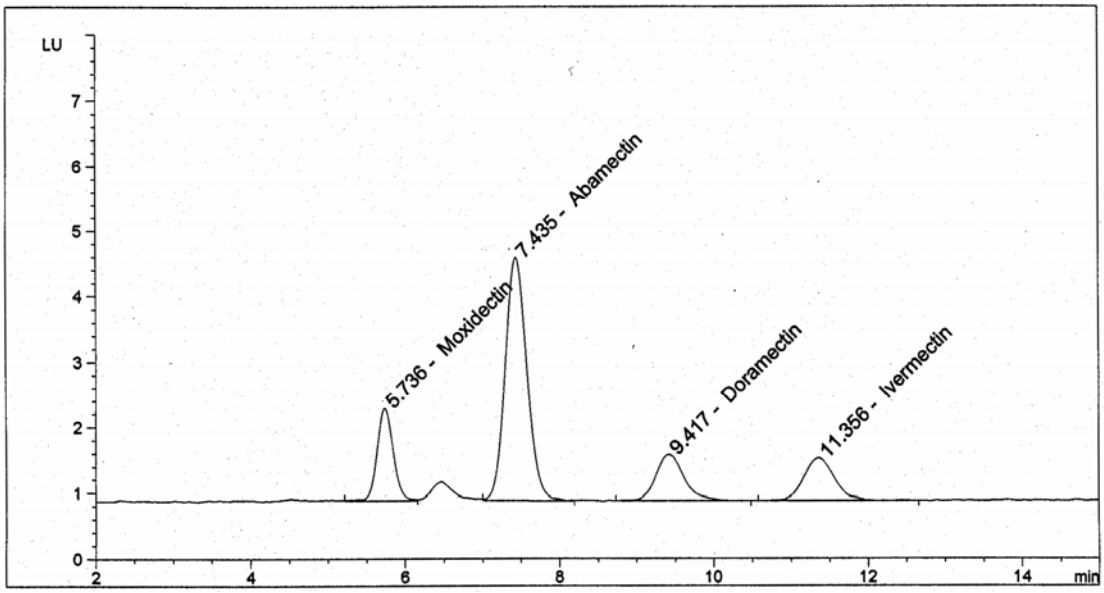
e. Run time: 15 min.

f. Detector settings:

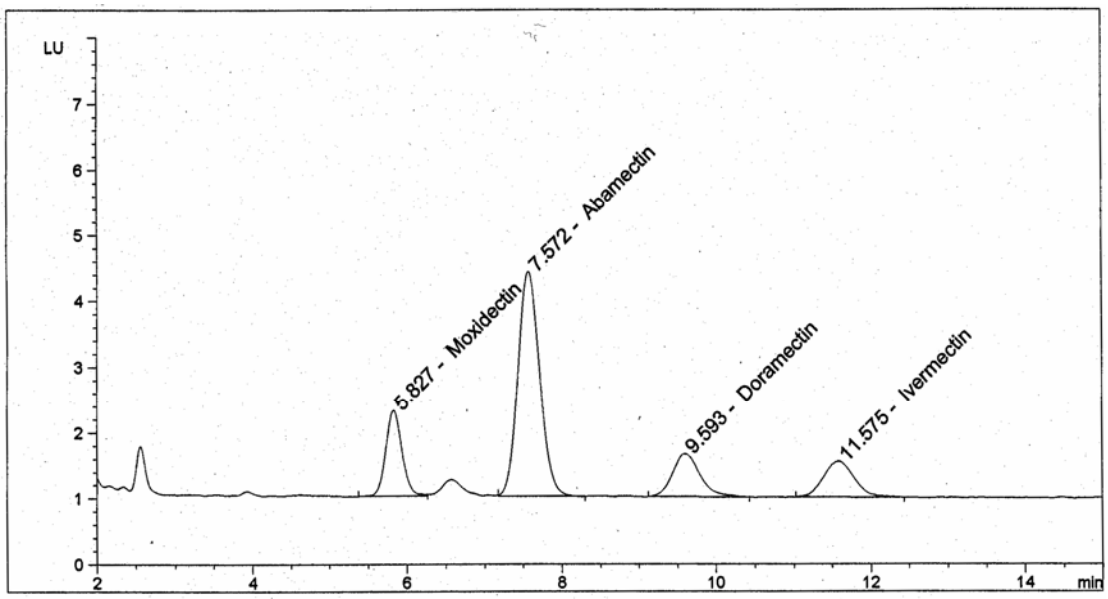
i. Excitation wavelength 365 ± 20 nm.

ii. Emission wavelength 465 ± 20 nm.

3. Sample Chromatograms



a. External Standard of Moxidectin (7.5 ppb), Abamectin (15 ppb), Doramectin (7.5 ppb), and Ivermectin (7.5 ppb).



b. Beef Liver fortified with Moxidectin (7.5 ppb), Abamectin (15 ppb), Doramectin (7.5 ppb), and Ivermectin (7.5 ppb).

G. CALCULATIONS

Quantitation is performed by measuring peak area. Each set is accompanied by an external standard curve at 0, 7.5,15, 30, and 60 ppb.

Measure peak area of abamectin, ivermectin, moxidectin, and doramectin peaks in the standards and calculate the peak area ratios.

$$\text{Peak Area Ratio} = \frac{\text{Moxidectin, Ivermectin or Doramectin Peak Area}}{\text{Abamectin Peak Area}}$$

Construct a linear regression line using the ratios and standard concentrations. The correlation coefficient should be >0.995.

The equation is $y = mx + b$, where

- $x =$ Ivermectin, Moxidectin, or Doramectin /Abamectin peak area ratio
- $y =$ Ivermectin, Moxidectin, or Doramectin concentration (ppb)
- $m =$ slope
- $b =$ y-intercept

Incurred tissue ivermectin, moxidectin, or doramectin concentrations should be calculated using this regression line.

Note: Peak heights may be substituted for peak areas if chromatographic peaks display sufficient symmetry.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, appropriate gloves, lab coat.

2. Hazards

Reagent	*Hazard*	*Recommended Safe Procedures*
Acetonitrile, Trifluoroacetic Anhydride, 1-methylimidazole	Flammable and corrosive, may cause skin or respiratory irritation.	Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.
Ivermectin Abamectin	Weak teratogen and possible mutagen	Handle with extreme caution.
Doramectin	Severe explosion hazard if in powdered form.	Handle with extreme caution.
Moxidectin	May cause skin or respiratory irritation. The toxic effects of this material have not been fully evaluated.	Work in a well ventilated area. Store material in a secure, dry, cool well ventilated room.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic solvents and Avermectin solutions	See above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well-ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range*	*Acceptable Recovery (%)*	*Acceptable Repeatability (CV (%))*
Ivermectin	≥ 7.5 ppb	60 -120	< 20
Doramectin	≥ 7.5 ppb	60 -120	< 20
Moxidectin	≥ 7.5 ppb	60 -120	< 20

Acceptability criteria:

a. Correlation coefficient ≥ 0.995.

b. Mean recovery for each species in range of 60 -120%.

c. Repeatability for each species < 20%.

d. No false positive or false negative results.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample weight	2.5 g ± 0.2 g.
b.	Alumina deactivation level	12%
c.	1-methylimidazole/acetonitrile volume	200 µL
d.	TFAA/acetonitrile volume	200 µL
e.	Condition of SPE column after sample loading (F.1.k.)	Do not allow SPE column to run dry
f.	Sample derivatization (F.1.o.)	In dark for a minimum of 15 minutes

3. Readiness To Perform (FSIS Training Plan)

a. Familiarization

i. Phase I: Standards-Duplicate standard curve on each of 3 consecutive days, which will include the following:

(a) 0 ppb

(b) 7.5 ppb

(c) 15 ppb

(d) 30 ppb

(e) 60 ppb

ii. Phase II: Fortified samples - 3 replicates at 0, 7.5, 15, 30 and 60 ppb of both liver and muscle of any applicable species (bovine, ovine, porcine, caprine and equine) over a period of 3 different days.

Note: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 8 samples fortified between 0 and 60 ppb. Four of the samples should be liver and 4 muscle. One of the 8 should be blank.

(b) Samples submitted by the Quality Assurance Manager (QAM) or supervisor.

(c) Letter from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to I.1.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One check sample per week per analyst when samples are analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I.1.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrices: Liver, muscle.

b. Species: Bovine, ovine, porcine, caprine, and equine.

c. Sample size: 16 oz. minimum

d. Condition on receipt: Frozen/semi-frozen

e. Sample storage:

i. Time: 6 months

ii. Condition: Frozen

6. Sample Set
   a. External standard curve at 0, 7.5, 15, 30, and 60 ppb
   b. Recovery
   c. Samples

7. Sensitivity
   a. Minimum proficiency level (MPL): 7.5 ppb.

J. WORKSHEET

The following worksheet is an example.

WorkSheet Printed

Sample Information	
Analyte Code:	990
Analyst Name:	
Analyst Code:	
Date Started:	
Date Compl'd:	
Check PPB:	
Rec PPB:	

Avermectin Screen Worksheet

Method

		PPB	MOX	ABA	DOR	IVR	M/A	D/A	I/A
Curve A	Blank	0					0	0	0
	A Std 1	7.5							
	A Std 2	15							
	A Std 3	30							
	A Std 4	60							
Curve B	Blank	0					0	0	0
	B Std 1	7.5							
	B Std 2	15							
	B Std 3	30							
	B Std 4	60							

Avermectin codes
Moxidectin: 992
Doramectin: 991
Ivermectin: 923

Regression Output:	A Curve		
	MOX	DOR	IVR
Y Intercept			
Slope			
R			
No. of Observations	5	5	5
Degrees of Freedom	3	3	3

Regression Output:	B Curve		
	MOX	DOR	IVR
Y Intercept			
Slope			
R			
No. of Observations	5	5	5
Degrees of Freedom	3	3	3

Analysis Parameters
500ng/ml Combined Std: Ivr, Dora, Moxi
Combined Std ILN
500 ng/ml Internal Std: Abamectin
Internal Std ILN

With 500 ng / mL conc.:
Spiking volume of 75uL = 15 ppb
Spiking volume of 150 uL = 30 ppb

Critical Control Points	
Sample weight:	2.5 +/- 0.2g
1-Methylimidazole volume:	200 ul +/- 10 ul
TFAA Volume:	200 ul +/- 10 ul
Final dilution volume:	2.5 ml

LC Parameters	
LC Column(ODS):	15cm X 4.6mm
Mobil Phase:	97:3,MeOH:H20
Flow Rate:	1.8 ml /min
Iver 1: Injection Volume =	
Iver 2: Injection Volume =	

Blank Used
Liver ID Number
Muscle ID Number

	Sample No	QAType	Curve	MOX	ABA	DOR	IVR	Moxidectin Results PPB	Moxidectin Results %Rec	Doramectin Results PPB	Doramectin Results %Rec	Ivermectin Results PPB	Ivermectin Results %Rec
1													
2													
3													
4													
5													
6													
7													
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K. APPENDIX

1. References

a. De Montigny, Pierre, Jung-sook, K. Shim and Pivnichny, J. V., J. Pharm. and Biomedical Anal., Vol. 8 No. 6, pp. 507-511, (1990).

b. Doherty, Steven J., Fox, Allen and Fink, David W., J.A.O.A.C. Vol. 73, No. 6, pp. 931-934, (1990).

c. Prabhu, Sunil V., Wherner, Teresa A., Egan, Richard S. and Tway, Patricia C., J. Agric. Food Chem., Vol. 39, pp. 2226-2230, (1991).

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Laboratory Quality Assurance Division.